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## Methylated DNA-binding protein 2 antisense inhibitors suppress tumourigenesis of human cancer cell lines *in vitro* and *in vivo*

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**Methylated DNA-binding protein 2 (MBD2) has been proposed to function both as a silencer of methylated genes and as a DNA demethylase. Our previous data indicated that knockdown of MBD2 inhibited tumourigenesis of human cancer lines and MBD2-deficient mice were recently shown to be resistant to intestinal tumourigenesis. MBD2 is an attractive anticancer target since MBD2-deficient mice were previously shown to be viable and fertile and knockdown of MBD2 was reported to have no effect on cellular growth parameters of non-transformed cells. In this paper we test the hypothesis that pharmacological inhibition of MBD2 inhibits cancer growth *in vivo* using human tumour lines implanted in mice as a model. We develop sequence-specific antisense inhibitors of MBD2 and we show that these agents inhibit anchorage-independent growth of human lung (A549) and colorectal (HCT116) cancer cell lines *in vitro* and tumourigenic growth of human cancer cell xenografts *in vivo*. MBD2 antisense oligonucleotide does not inhibit the growth of normal and transformed cell lines and does not alter cell cycle parameters *in vitro* and does not exhibit overt toxicity *in vivo* in comparison with a scrambled control oligonucleotide, as determined by measuring body mass, blood cell parameters and liver and kidney enzymes. Our data provide a proof of principle that MBD2 is a new anticancer target and that pharmacological inhibition of MBD2 by agents such as the antisense inhibitors described in this paper is a potential new anticancer therapy, which in contrast to the vast majority of current approaches does not target normal progression of the cell cycle.**

### Introduction

Aberrations in DNA methylation patterns are commonly seen in tumours (1) and the DNA methylation machinery has been proposed as a potential target for anticancer therapy (2,3). To date, genetic (4,5) and pharmacological data (6) support the hypothesis that inhibition of DNA methyltransferase 1, the enzyme responsible for replicating the DNA methylation pattern, can block tumourigenesis. Methylated DNA-binding proteins (MBDs) constitute a family of proteins that recognize methylated DNA and are responsible for creating, maintaining and interacting with the epigenome (7,8). One of the methylated binding proteins, MBD2, has been described as both a

transcriptional repressor (9,10) and as a demethylase (11–16). The demethylase activity of MBD2 is, however, controversial (9,10,17). Although the exact role of MBD2 in gene expression is still unclear, there is strong evidence suggesting that MBD2 is critical for tumourigenesis. Evidence of increased MBD2 expression in tumours and cancer cells has been observed (18–20) and its expression in mammary tumours correlates with tumour size (18), although decreased expression has also been reported (21,22). Recent data suggested that MBD2 was required for tumourigenesis in human cancer cell lines (23) and MBD2-deficient mice were shown to be resistant to intestinal neoplasia (24). Since it has also been shown that MBD2 knock-out mice are viable (25) and that knockdown of MBD2 does not inhibit normal cell growth parameters (23), in this paper we tested the attractive hypothesis that pharmacological agents that reduce MBD2 could selectively inhibit human cancer cells grown as tumours *in vivo* in nude mice without affecting the viability of non-cancerous tissue. Although MBD2 deficiency rendered mice resistant to tumourigenesis, it was unknown whether inhibition of MBD2 would inhibit established tumours or whether MBD2 was required just to initiate the tumourigenic state and not for maintenance of tumourigenicity. Our previous data have shown that an adenoviral vector expressing the cDNA for MBD2 in the antisense orientation inhibited anchorage-independent growth of human cancer cells *in vitro* and had some effect *in vivo*, supporting the hypothesis that MBD2 is required for the maintenance of tumourigenicity. However, the effect of the MBD2 antisense expressing adenoviral vector *in vivo* was partial. It was not clear whether the incomplete anti-tumourigenic effect was a result of inefficient delivery of the adenoviral antisense expression vector *in vivo* or because the target is not essential for tumourigenesis *in vivo*. It was apparent therefore that in order to address the question of whether inhibition of MBD2 is a potential anticancer therapeutic approach we needed to inhibit MBD2 in human tumour cells by an efficacious pharmacological agent *in vivo*. We show here the selection of sequence-specific second generation MBD2 antisense oligonucleotides which knockdown MBD2 and inhibit the growth of human cancer cells *in vitro* and *in vivo* when the tumours are implanted into nude mice. These MBD2 antisense inhibitors do not have anti-proliferative side effects *in vivo*. We propose that pharmacological inhibitors of MBD2 such as those presented in this paper are potential novel anticancer therapeutic agents, which target tumourigenesis while bypassing the cell proliferation machinery and should thus avoid the side-effects caused by common anticancer agents. MBD2 inhibitors also provide opportunities for understanding the distinct processes in cancer initiation and progression that are controlled by MBD2.

### Materials and methods

#### Design of oligonucleotide sequences

Antisense DNA oligonucleotides (oligos) were designed to hybridize to human MBD2 cDNAs: A1–A3, A7–A10 and 12258/9 (11) and A5–A6 (7).

Abbreviations: MBDs, methylated DNA-binding proteins; MBD2, methylated DNA-binding protein 2; PS, phosphorothioate.

The sequences of the oligos used in this paper are as follows: A1, 5'-GGCAATC-CATCTCTTCC-3'; A2, 5'-CTTCCTCTTCTTCCATC-3'; A3, 5'-CAA-CAGTATTTCCAGGT-3'; A4, 5'-TGAGCCTCTTCTCCAG-3'; A5, 5'-ATCCAGCCCCCTCCAG-3'; A6, 5'-CACTCTCCCCCTCCCT-3'; A7, 5'-TCAACAGTATTTCCAGGTA-3'; A10, 5'-UCAACAGTATTTCCAGGUA-3'; A11, 5'-AUGGACCCCTTATGACAACU-3'; 12258, 5'-AGTAGA-CATCGCTCTTCCAGCAC-3'; 12259, 5'-TTCCACAGCAGCGAGACTTGCCCT-3'; 1582, CGATTCAATCTCACCTCTC-3'. Oligos A1-A5 have a phosphorothioate (PS) backbone, while A7, 12258 and 12259 have a mixed phosphodiester and PS backbone, A7 contains an inverted thymine base at the 3'-end and A10 and A11 have a complete PS backbone, with a 2'-O-methyl modification of the ribose on the first four and last four bases. Mixed backbone oligos retain much of the nuclease resistance of PS oligos, with a reduction in toxicity (26). Finally, the A10 oligo used for much of the *in vitro* and all of the *in vivo* trials has a PS backbone, with the first four and last four bases being 2'-O-methyl modified RNA bases. This structure lends itself to RNase H-mediated degradation of target RNA, with greater *in vivo* bioavailability (27). Control oligos A11 and 11899 are the reverse sequences of A10 and 12259, respectively, with oligo 1582 used as a non-specific control for the rest of the experiments.

Oligos were selected to avoid dimers and stem-loop structures, as well as CpG dinucleotides (28). A4-A10 include the TCCC tetranucleotide motif shown to increase the probability of RNase H-mediated RNA destruction (29). After synthesis, the oligos were cleared of contaminants by desalting and gel purification (A1-A6), HPLC (A7, 12258 and 12259) or ion exchange HPLC (A10 and A11). Oligo synthesis was performed by: A1-A7, Oligos Etc. (Wilsonville, OR); 11899, 12258 and 12259, Sequitur (Natick, MA); A10 and A11, Integrated DNA Technologies (Coralville, IA).

**Oligonucleotide transfection and analysis of MBD2 mRNA and MBD2 protein**  
A549 human non-small cell lung carcinoma (ATCC no. CCL-185), HCT116 human colon carcinoma cells (ATCC no. CCL-247) and MRHF human foreskin fibroblasts (Biowhittaker, Walkersville, MD) were cultured free of antibiotics in the recommended media to facilitate oligo transfection and plated at a density of  $3 \times 10^5$  per 10 cm plate 24-48 h before transfection. Oligos were added to cells at a final concentration of 20, 50 or 200 nM using either 6.25 µl/ml Lipofectin for A549 cells or 4 µl/ml Lipofectamine (Invitrogen, Carlsbad, CA) for HCT116 cells as lipid carriers. Transfection was repeated after 24 h, cells were harvested with TRIzol (Invitrogen), total RNA was prepared according to the manufacturer's recommendations and was subjected to gel electrophoresis, northern blot transfer onto Hybond-N<sup>+</sup> membrane (Amersham Pharmacia Biotech) according to the manufacturer's recommendations and hybridization with a 1.3 kb *MBD2b* cDNA probe (11) in a 10 ml hybridization solution containing 7% SDS, 0.5 M sodium phosphate, pH 6.8, and 1 mM EDTA at 68°C for 4 h. Following washing and exposure to a phosphorimaging plate, the membrane was stripped and hybridized to a <sup>32</sup>P-labelled 18S rRNA probe as previously described (30). The signals for *MBD2* and 18S rRNA were quantified by densitometry and the intensity of the *MBD2* signal was normalized to the signal obtained for 18S rRNA. A549 cells were harvested after a single treatment with oligo, nuclear extracts prepared as previously described (31) were resolved by electrophoresis and membranes were probed with a 1:200 dilution of a monoclonal anti-MBD2 antibody (Imgenex, San Diego, CA) in 0.05 M Tris-0.2 M NaCl, pH 7.6, containing 0.5% Tween 20 and 5% milk overnight at 4°C. The blot was then reacted with a secondary anti-mouse monoclonal antibody at a 1:20000 dilution and the band developed with an ECL kit from Amersham Pharmacia Biotech. To control for equal loading, β-actin western blots were performed using a 1:5000 dilution of anti-β-actin monoclonal antibody (Sigma catalogue no. A-5316), followed by mouse secondary antibody as just described for MBD2. Primers used for RT-PCR analysis of *DNMT1* and the sequences of *DNMT1* antisense ODN MG88 and the mismatch control MG208 were previously described (33).

#### Anchorage-independent and -dependent growth analyses

To determine anchorage-independent growth on soft agar, 3000 live cells treated with a single dose of antisense or control oligo for 24 h were seeded into soft agar and plated in triplicate in a 6-well plate for 21 days as previously described (34). The number of colonies (>10 cells/colony) in five random fields (40×) per well, throughout all planes of the triplicate wells, was counted after 21 days under a microscope.

For anchorage-dependent cell growth experiments, A549 and HCT116 cells were plated at a concentration of 50 000 cells/dish. After 24 h, cells were treated with 200 nM control or *MBD2* antisense oligo. Treatments were repeated after 24 h and cells were harvested 24, 48 and 72 h after the second treatment (days 1, 2 and 3, respectively). To determine cell cycle kinetics, the cells were fixed for 18 h in 70% ethanol at 4°C and treated with propidium iodide to label DNA (35). Twenty-thousand cells were sorted in triplicate for

DNA content in a Becton Dickinson (Franklin Lakes, NJ) FACScan cell sorter and data were acquired by the LYSIS II program (36).

#### Analysis of hematological and biochemical parameters

Analysis of haematological values and serum biochemical parameters was performed by McGill University Animal Resources Centre diagnostic and research support service using standard procedures.

## Results

### Selection of specific MBD2 antisense oligonucleotides

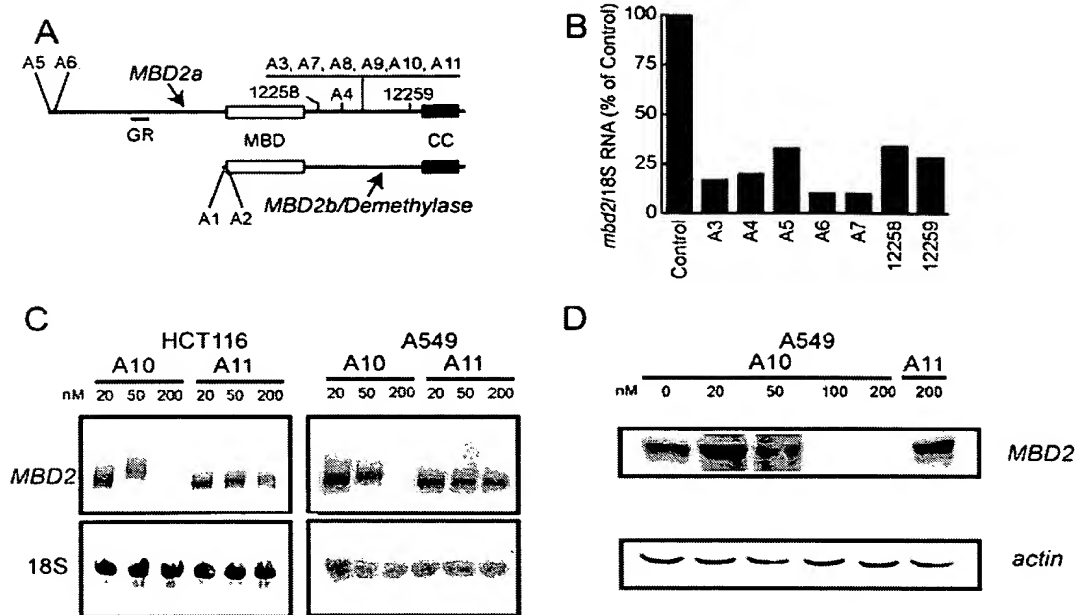
To test the hypothesis that pharmacological inhibition of MBD2 is in principle a novel approach to human anticancer therapy, we designed antisense oligos that could be administered as pharmacological agents *in vivo* using human tumours implanted in nude mice as a model. The general rules that guided our design of antisense oligos are described in Materials and methods. Following initial screening of several antisense oligos to human *MBD2* cDNA (Figure 1A) showing varying degrees of *MBD2* mRNA inhibition at the 200 nM concentration relative to a control oligonucleotide, we selected A7, which exhibited up to 90% inhibition (Figure 1B). The A10 sequence is highly conserved between mouse and human and there is only one base mismatch between the human and mouse sequence. A7 was further modified by positioning a 2'-O-methyl modification on the ribose of the first and last 4 nt and was designated A10. This structure enables RNase H-mediated degradation of target RNA, with greater *in vivo* bioavailability (27). A11, the reverse sequence of A10, served as a control. Specific and dose-dependent inhibition of target *MBD2* mRNA was observed in both A549 and HCT116 cells treated with A10 (Figure 1C) in comparison with the A11 control. Dose-dependent knockdown of MBD2 protein in A549 cells was confirmed by western blot analysis (Figure 1D).

### MBD2 antisense inhibitors reduce anchorage-independent growth of human cancer cell lines

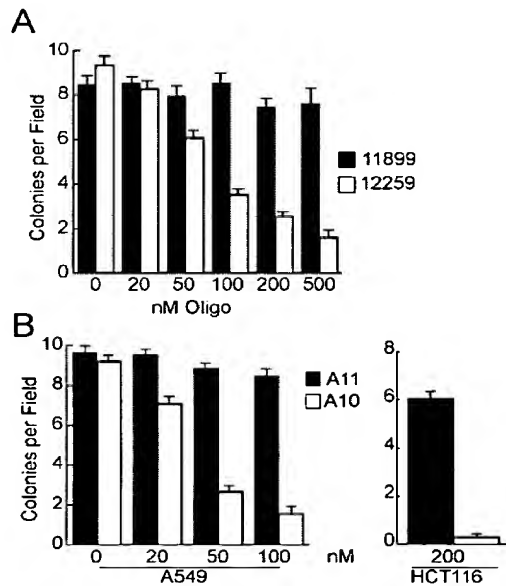
We first tested the hypothesis that MBD2 antisense oligos inhibit anchorage-independent growth of human cells on soft agar, which is an indicator of the tumorigenic potential of cancer cells (37). Both 12259 and A10 *MBD2* antisense oligos inhibited the anchorage-independent growth of A549 cells in comparison with the 11899 and A11 reverse controls (Figure 2A and B) in a dose-dependent manner. 12259 is somewhat less effective than A10 in its inhibition of *MBD2* mRNA (Figure 1B) as well as anchorage-independent growth (Figure 2A), we therefore focused on A10. A10 also inhibited anchorage-independent growth of HCT116 cells using a 200 nM dose, suggesting that this inhibition is not unique to A549 cells (Figure 2B). The fact that the same result was observed with two independent sets of antisense oligos that bear different backbone modifications (12259 and A10) supports the hypothesis that inhibition of anchorage-independent growth of A549 cells is sequence specific and is a consequence of the knockdown of MBD2 rather than some idiosyncrasies of the specific antisense oligonucleotide.

### Inhibition of tumour implantation *in vivo* by MBD2 antisense oligonucleotides

Since *MBD2* antisense treatment prohibited anchorage-independent growth of both colon- and lung-derived tumour cells, we determined whether such a treatment could inhibit the ability of tumour cells to implant *in vivo*. One property of tumour cells is their ability to implant as xenografts *in vivo*



**Fig. 1.** Screening of antisense oligonucleotides to *MBD2*. (A) cDNA of human *MBD2a* (upper) and *MBD2b/Demethylase* (43) and locations of antisense oligonucleotides. GR indicates glycine-arginine repeat region, CC is a coiled-coil domain, MBD is the consensus methylated DNA-binding domain. (B) Normalized *MBD2* mRNA levels in A549 cells treated with 200 nM indicated oligonucleotides. (C) Representative northern blots measuring *MBD2* expression in A549 and HCT116 cells following 48 h treatment with the indicated modified antisense oligos (44). (D) Western blot of nuclear extracts showing a reduction in MBD2 protein in A10 antisense versus A11 control treated A549 cells. The western blots were reacted with  $\beta$ -actin antibody to control for protein loading.



**Fig. 2.** Antisense oligos to *MBD2* inhibit contact-independent growth. The graphs show dose-dependent reduction in A549 colony growth in soft agar following a single treatment with 12259 (A) or A10 (B, left) antisense oligos (open bars). Reverse sequence controls (11899/A11) are shown as solid bars. (B, right) Antisense A10 reduces colony growth of HCT116 cells by ~95%. Triplicate experiments were pooled, with five random fields chosen per well. Error bars are SEMs.

as well as migrate and metastasize into non-cognate tissues. To differentiate between the role of MBD2 in tumour growth *in vivo* and the implantation process *per se*, we first treated the cells with *MBD2* antisense oligo *in vitro* and then implanted them *in vivo*. The animals were not treated with oligonucleotides. Naïve cells of both A549 lung cancer and HCT116 colorectal cancer lines showed rapid tumour growth when implanted into BALB/c nude mice (data not shown). Samples of  $2.5 \times 10^6$  of A549 or HCT116 cells (8 mice/treatment group) were treated for 48 h *in vitro* with either 200 nM A10 or the control A11 oligonucleotide and were injected s.c. into the right flank of female BALB/cAnNCrl-nuBR nude mice (5–6 weeks old; Charles River). Whereas palpable tumours formed rapidly from A11 control treated cells, this process was slowed in A10-treated cells (Figure 3A and B). Effects on tumour burden were even more dramatic for HCT116 cells, with all six of the A11 group showing measurable tumours, but only one of nine of the antisense group. Growth rate was reduced for A10-treated cells, as was final tumour mass (54% for A549, 95% for HCT116 cells; Figure 3A and B, insets). These results are similar to the soft agar experiment, in which pretreatment of the cells inhibited their ability to grow on soft agar even in the absence of any further treatment, suggesting that seeding of cancer cells in an anchorage-deprived environment requires MBD2. Since the cells were treated only once *in vitro* and were not further treated *in vivo*, our results are consistent with the hypothesis that MBD2 is required for the first stage of implantation of tumours in a foreign environment *in vivo*. HCT116 cells are more sensitive to treatment *in vitro* with A10 than A549 cells. We do not

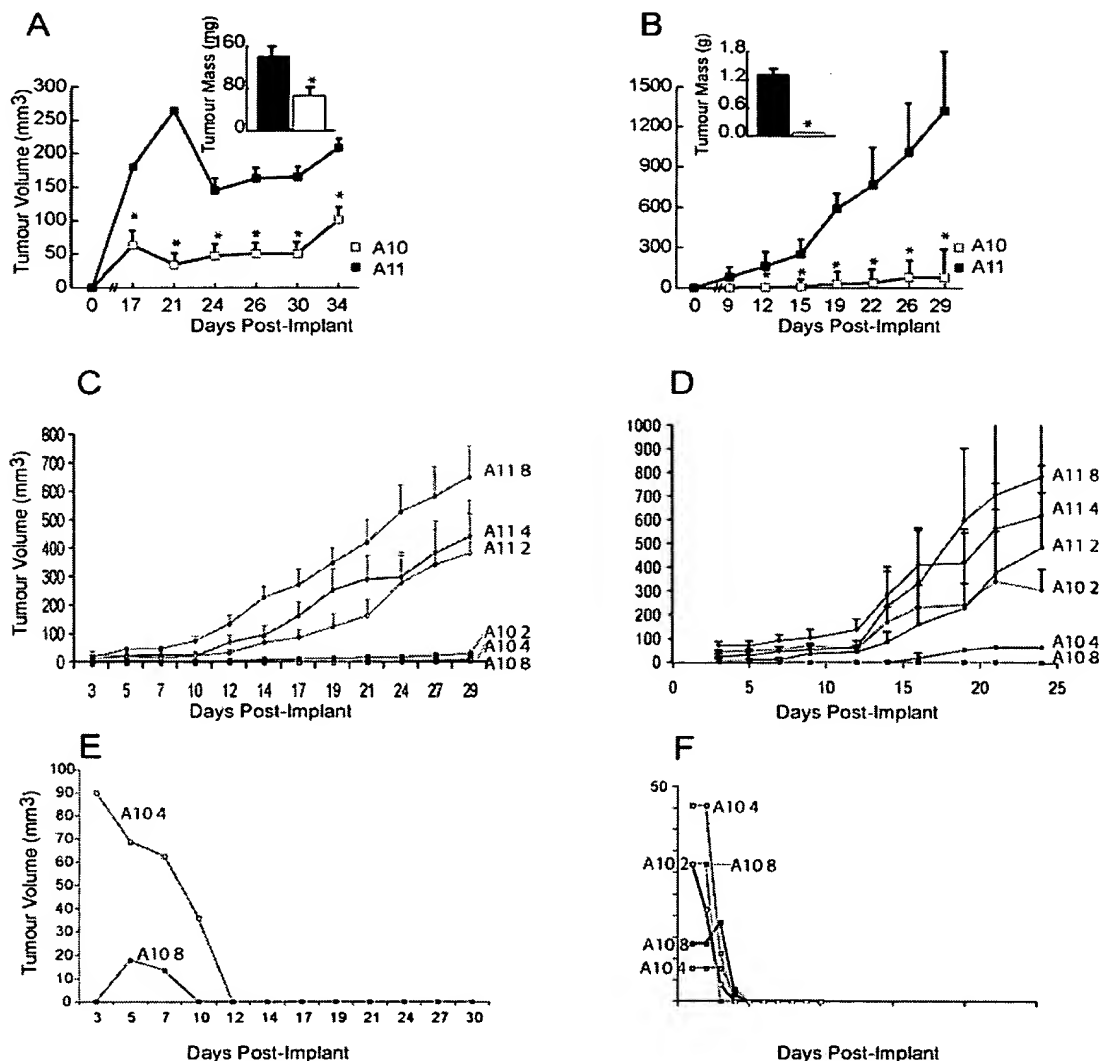


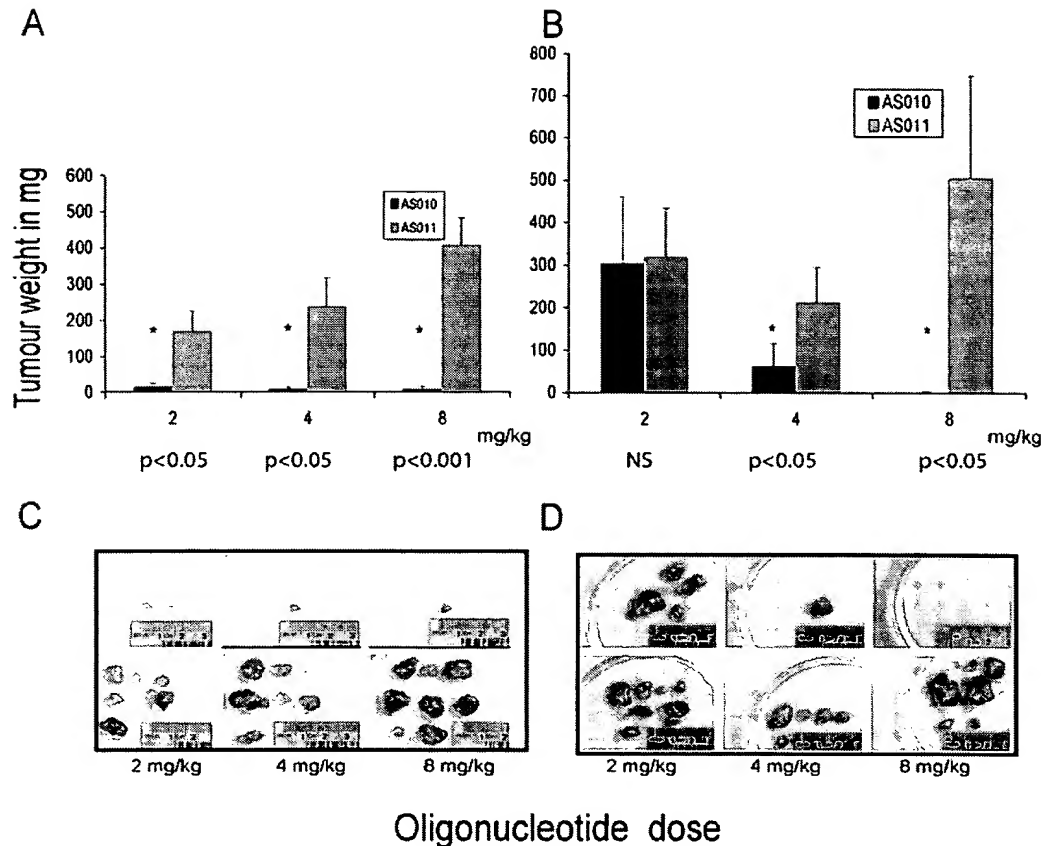
Fig. 3. Antisense oligos inhibit xenograft tumour growth. *In vitro* treatment of A549 (A) or HCT116 (B) cells with A10 antisense oligo reduces tumour growth when implanted into nude mice ( $n = 6-8$  mice/group). The graphs show average tumour volume and mass (inset)  $\pm$  SEM. A two-sided Student's *t*-test was performed to determine whether the A10 and A11 treatment groups are significantly different. Stars above the bars indicate statistical significance at  $P < 0.05$ . Chronic *in vivo* dosing of A10 inhibits growth of naive A549 (C) and HCT116 (D) cells implanted into nude mice in a dose-dependent fashion (the treatment, AS10 or AS11, and dose in mg are indicated next to the line) (44). Several mice showed complete tumour regression (E and F). Each line represents one tumour (the treatment and dose are indicated next to the line). Two A549 tumours (E) and five HCT116 (F) tumours underwent regression during the experiment.

know whether this is caused by the somewhat more effective inhibition of *MBD2* mRNA by A10 in HCT116 cells (Figure 1C) or whether HCT116 and A549 cells have different requirements for *MBD2* for their ability to implant *in vivo* and to grow in an anchorage-independent manner.

#### *MBD2* antisense oligonucleotide inhibits tumour growth *in vivo*

We then determined whether pharmacological administration of A10 inhibits the growth of pre-implanted naive human cancer cells in nude mice. Samples of  $2.5 \times 10^6$  naive A549 or HCT116 cells were injected s.c. into BALB/cAnNCrI-nuBR nude mice. Three days post-implantation, when solid tumours were already visible, the mice (8 per group) were injected via

the tail vein three times per week with control or antisense oligo, at doses of 2, 4 or 8 mg/kg dissolved in 100  $\mu$ l sterile phosphate-buffered saline. The mice were monitored for tumour growth three times per week and tumour volume was estimated by using  $V = (L \times W^2) \times 0.5$ , where *L* is the length and *W* is the width of a xenograft (38), and observed for cachexia, lethargy, lesions and other signs of toxicity. The mice were killed after 24 or 29 days by exsanguination and their blood was analysed for liver and kidney function as well as haemocytology. A549 cells were more sensitive to systemic antisense treatment than *in vitro* treatment. All three doses of A10 showed significant inhibitory effects on tumour growth (Figure 3C), with complete tumour regression seen in



**Fig. 4.** Tumour mass is significantly reduced by *MBD2* antisense treatment. Tumours were excised and weighed. Average tumour mass is plotted with the SEM in (A) (A549) and (B) (HCT116). The tumours were photographed (C, A549 experiment, upper panel A10, bottom panel A11; D, HCT116 experiment, upper panel A10, bottom panel A11) at death. A two-sided Student's *t*-test was performed to determine whether the A10 and A11 treatment groups are significantly different. The *P* values are indicated under the respective bar graphs. Stars above the bars indicate statistical significance. NS, not significant.

two mice dosed with 8 mg/kg (Figure 3E). One mouse in this same antisense group died 17 days post-implantation, but the cause of death was unclear. Numbers of tumour-free mice for the 2, 4 and 8 mg/kg groups were 5/8, 7/8 and 7/8 for A10 and 1/8, 1/8 and 0/8 for A11 treatments. The mice were killed after 29 days and the tumours were weighed (Figure 4A) and photographed (Figure 4C). Thus, *MBD2* antisense treatment essentially eliminated the ability of A549 cells to grow as tumour xenografts in nude mice. *MBD2* antisense inhibition of tumour growth is not limited to human lung non-small cell carcinoma since A10 also reduced tumour growth of HCT116 colon cancer cells in nude mice (Figure 3D). HCT116 cells appeared to be extremely sensitive to reductions in *MBD2*, as indicated by the complete regression of tumours in some mice at each dose (Figure 3F). However, the dose required for maximal effect on HCT116 cells (8 mg/kg) was higher than the dose required for A549 cells (maximal effect is achieved already at 2 mg/kg). Numbers of tumour-free mice for the 2, 4 and 8 mg/kg groups were 5/8, 7/8 and 8/8 for A10 and 1/8, 1/6 and 1/8 for A11 treatments. Two mice in the 4 mg/kg A11 group died of cachexia, perhaps due to tumour burden. None of the antisense oligo-treated mice implanted with HCT116 cells died before being killed. A dose-dependent reduction in tumour mass with

A10 antisense treatment was observed when the tumour mass was determined following killing of the animals (Figure 4B and D). In summary, our data suggest that *MBD2* is necessary for tumour growth *in vivo* in addition to being required for tumour implantation.

#### *MBD2 antisense oligo treatment does not inhibit cell growth and viability of either normal or cancer cells under anchorage-dependent conditions*

As non-specific effects on normal cell viability and growth often hinder the therapeutic potential of many anticancer agents, we investigated the impact of A10 on cell proliferation of normal human cells. An antisense oligo to *MBD2* proved to be relatively innocuous to the growth of normal MRHF human foreskin fibroblasts. Dosing regimens of A10 for the same time period of 48 h that proved inhibitory to cancer cell colony formation and xenograft growth (Figures 2 and 3A) had little effect on contact-dependent growth of MRHF cells relative to A11 or mock treatment (Figure 5A). Cell viability, as measured by trypan blue exclusion, was ~100% over days 1–3 (data not shown). We also determined the effect that an *MBD2* antisense oligo might have on the A549 and HCT116 cell lines (Figure 5B and C). Surprisingly, in light of the strong inhibitory effects

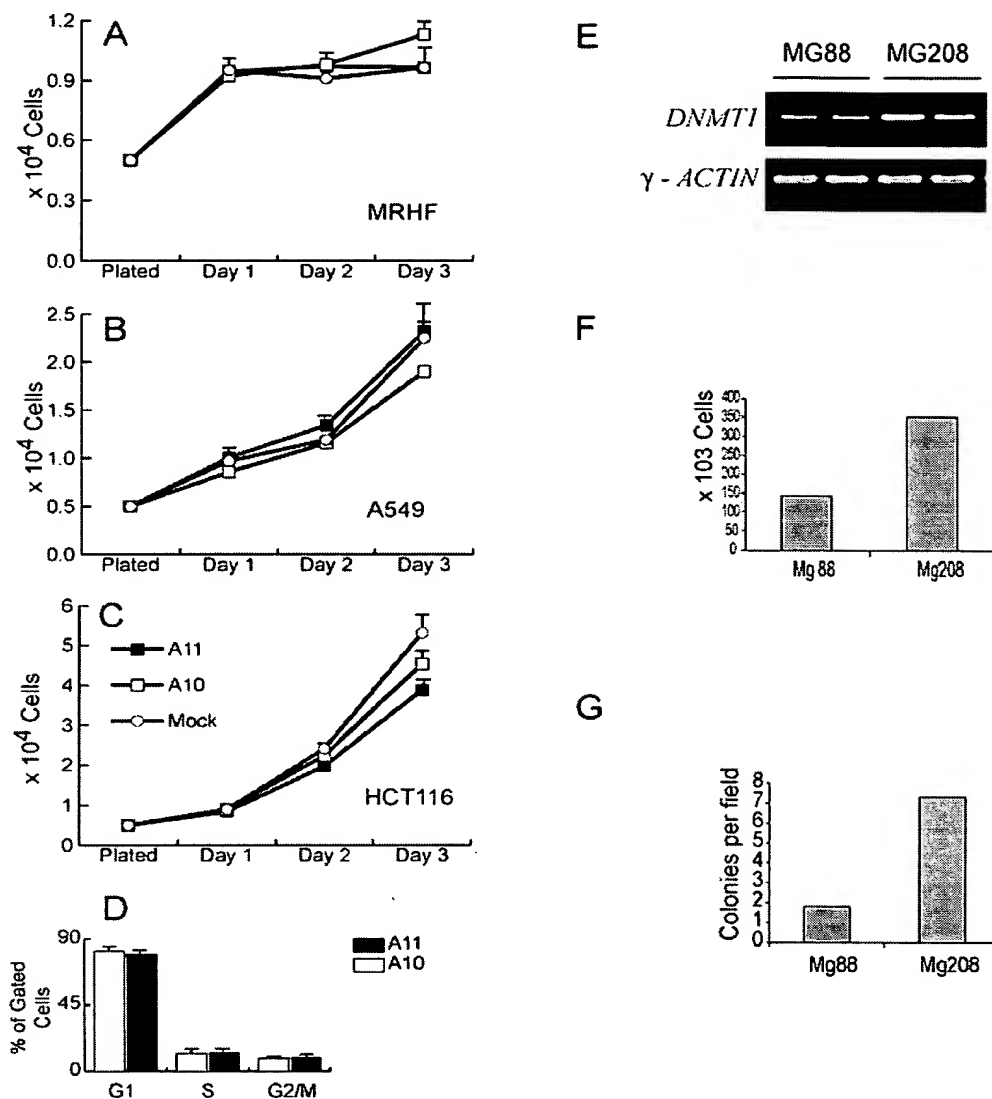
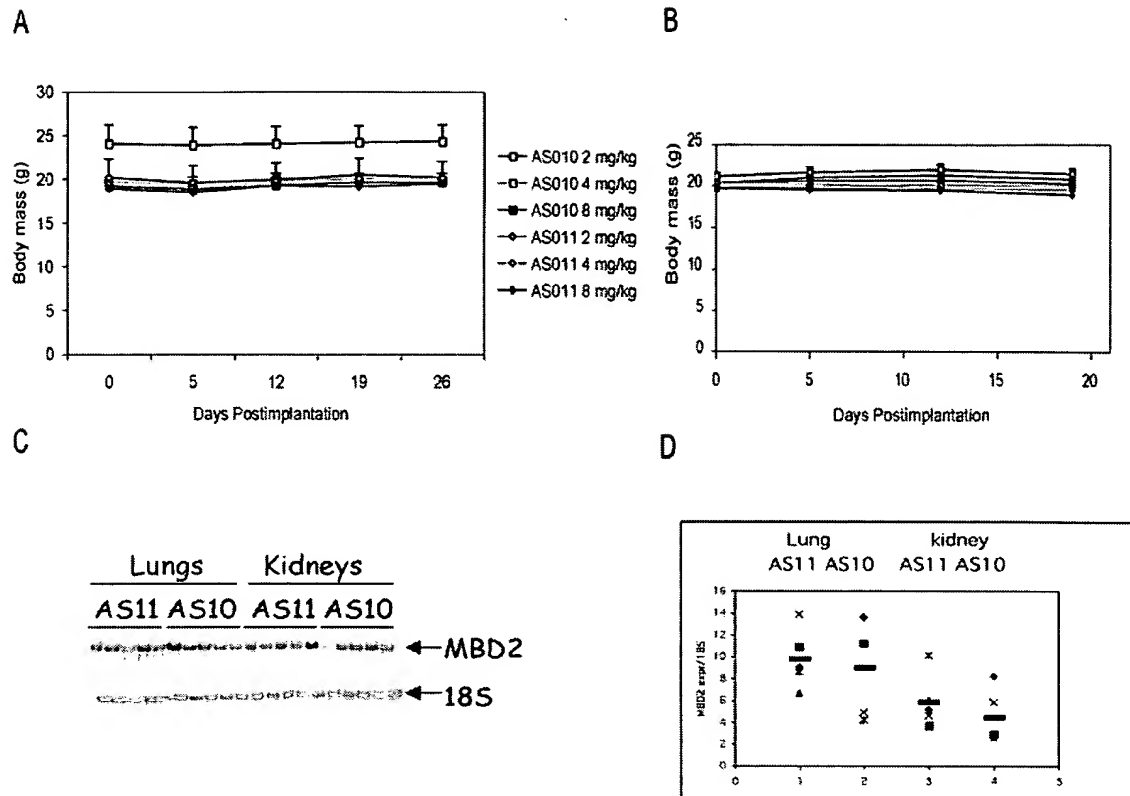


Fig. 5. *MBD2* antisense oligo A10 is non-toxic *in vitro*. Graphs show growth curves of MRHF (A), A549 (B) and HCT116 cells (C) following 2 days of treatment with A10 (open squares) or A11 (solid squares) or mock transfection (open circles) (averages of three replicates  $\pm$  SEM). (D) Oligo A10 has no effect on cell cycle progression of A549 cells (error bars are SEM for triplicate transfections). (E) RT-PCR analysis of *DNMT1* mRNA in A549 cells treated with either 200 nM MG88 or the control MG208 (bearing a 4 base mismatch with *DNMT1* mRNA) for 48 h. MG88 treatment for 48 h reduces (F) anchorage-dependent and (G) anchorage-independent growth of A549 cells.

on tumorigenicity in these cells, A10 had minimal impact on the ability of the tumour cells to grow on a semi-solid support (Figure 5B and C). For A549 and HCT116 cells viability remained  $>90\%$  for the A10, A11 and mock treatment groups, with no differences between groups. There was no indication of cell death or apoptosis. Finally, we treated A549 cells with 200 nM A10 or A11 and harvested the cells to look at progression through the cell cycle. FACS analysis revealed that cohorts of cells remained unchanged throughout the cell cycle (Figure 5D). These data indicate that the oligos are neither cytotoxic nor cytostatic for growing cells (tumour or non-neoplastic) and that they do not induce apoptotic or necrotic mechanisms. The fact that A10 treatment does not impact on either growth or cell

cycle kinetics excludes the possibility that the antisense DNA acts by an interferon-mediated pathway, which should result in cell cycle arrest (39,40).

To demonstrate that A10 exhibits a gene-specific effect rather than a non-specific 'double-stranded RNA-DNA' effect on tumour cells, we compared the effect that A10 had on growth of A549 cells to the effects of MG88, a well-characterized antisense inhibitor of *DNMT1* (Figure 5E) on the same cells. MG88 is well documented to inhibit tumorigenesis *in vitro* similar to A10 (41). However, different to A10, MG88 has a dramatic effect on cell growth after 48 h treatment (Figure 5F). This difference in the effects of these two antisense oligos on cell growth parameters highlights the



**Fig. 6.** *MBD2* antisense treatment does not affect animal body mass. (A and B) The animals from the experiments presented in Figure 4 (A, A549 experiment; B, HCT116 experiment) were weighed at the indicated time points and the average weight per treatment group is indicated in  $g \pm SEM$ . (C) Five animals were injected with either A10 or A11 at a dose of 8 mg/kg at an interval of 48 h, were killed 48 h after the second injection and RNA was prepared from the lungs and kidneys. The RNA was subjected to northern blot analysis and hybridization with either an *MBD2* or an 18S rRNA probe as indicated. (D) The intensities of the signals for *MBD2* mRNA normalized for 18S rRNA were determined by densitometry. The horizontal line indicates the average per group.

fundamental difference between *DNMT1* and *MBD2* antisense inhibitors, supporting the conclusion that *MBD2* antisense effects are gene specific. It also highlights the uniqueness of *MBD2* inhibitors amongst anticancer targets, which usually target the cell cycle machinery, like MG88. Our data showing that *MBD2* knockdown has no effect on cell growth agrees with the normal development, viability and fertility of *mbd2*<sup>-/-</sup> mice (24). In summary, *MBD2* has a distinct and unique role in tumourigenesis in that it is required for tumourigenesis *in vivo* and *in vitro* but not for cell growth.

Since the A10 antisense oligo is highly complementary to the murine *mbd2* mRNA sequence (there is only one base mismatch between the human and mouse sequence at this position), we determined whether A10 had a specific toxic effect on proliferating tissues *in vivo*. We first monitored animal weight throughout the experiment. The results shown in Figure 6 demonstrate that *MBD2* antisense oligonucleotides had no effect on animal weight at any of the concentrations used in this experiment. Blood samples were obtained from treated mice at death and screened for various markers of *in vivo* toxicity (Table I). Throughout the various dosing concentrations no differences were seen between A10 and A11 for haematological measurements, indicating that blood cell precursors, which are often sensitive to chemotherapeutic regimens, were largely unaffected by serial dosing with antisense oligos. Platelet

numbers were, however, increased above normal levels, regardless of treatment or dose; this is probably caused by the multiple tail vein injections. Urea, creatinine, aspartate aminotransferase and alanine aminotransferase, indices of liver, muscle and kidney damage, similarly showed no negative effects from *in vivo* treatment. Swelling of the inguinal lymph nodes was a non-specific but dose-dependent effect of the oligos that occurred in several mice treated with 4 or 8 mg/kg.

Although the A10 target is almost identical to the mouse *mbd2* mRNA sequence, which should have resulted in knockdown of the murine message, it is not clear whether the lack of effect is a consequence of limited delivery or the completely innocuous consequences of *MBD2* knockdown. It is hard to determine the extent of knockdown of *MBD2* in living tissues since the tissue might include different cell types with different ODN permeabilities. Acute treatment of mice with either A10 or A11 as a control revealed a bimodal response in *MBD2* mRNA levels (Figure 6B). Two of the five treated mice had reduced *MBD2* mRNA in the kidney and three of the five treated mice had reduced *MBD2* mRNA in the lungs relative to A11-treated controls. The complexity of assessing the significance of these reductions in living organs precludes us from arriving at a definite answer. Further extensive experiments are required to define the extent of *MBD2* inhibition in normal tissues. However, taken together with the previously published



Table I. *MBD2* antisense oligo is non-toxic *in vivo*

	2 mg/kg		4 mg/kg		8 mg/kg	
	A10	A11	A10	A11	A10	A11
Hematocrit	0.44 ± 0.01	0.45 ± 0.01	0.46 ± 0.01	0.44 ± 0.01	0.44 ± 0.01	0.45 ± 0.01
Hemoglobin (g/l)	152.88 ± 4.3	154.75 ± 3.3	156.81 ± 3.2	149.46 ± 2.0	153.02 ± 1.6	154.81 ± 3.1
RBC (10 <sup>12</sup> /l)	9.07 ± 0.2	9.11 ± 0.3	9.32 ± 0.2	8.78 ± 0.2	9.14 ± 0.1	9.43 ± 0.3
MCV (fl)	48.88 ± 0.5	49.56 ± 0.4	49.44 ± 0.6	49.69 ± 0.3	48.41 ± 0.3	48.19 ± 0.4
MCH (pg)	16.85 ± 0.2	17.02 ± 0.2	16.87 ± 0.2	17.06 ± 0.2	16.83 ± 0.2	16.47 ± 0.3
MCHC (g/l)	345.19 ± 3.2	345.32 ± 3.4	341.13 ± 2.4	344.23 ± 3.7	348.35 ± 2.2	343.22 ± 5.0
WBC (10 <sup>9</sup> /l)	7.19 ± 0.6	6.04 ± 0.7	8.23 ± 0.5	6.28 ± 1.0	8.04 ± 0.7	9.23 ± 0.7
Platelets (10 <sup>9</sup> /l)	821.00 ± 119.5	662.13 ± 81.3	630.81 ± 89.6	796.79 ± 84.0	608.54 ± 86.9	591.90 ± 64.7
Urea (mM)	7.8 ± 0.5	7.7 ± 0.2	8.9 ± 1.1	7.6 ± 0.6	8.0 ± 0.6	9.1 ± 0.7
Creatinine (mM)	39.4 ± 1.2	40.8 ± 0.8	40.5 ± 1.3	41.0 ± 1.2	40.3 ± 1.4	38.5 ± 1.5
Albumin (g/l)	37.9 ± 0.4	38.2 ± 0.4	37.5 ± 0.5	38.1 ± 0.8	37.6 ± 0.8	36.4 ± 0.6
ALT (U/l)	82.1 ± 19.9	83.2 ± 18.0	113.6 ± 33.5	79.7 ± 19.1	92.1 ± 27.2	93.9 ± 24.0
AST (U/l)	216.3 ± 31.8	165.1 ± 25.3	256.3 ± 79.4	237.8 ± 72.2	297.1 ± 90.7	282.6 ± 60.5

Blood samples were drawn from xenograft mice at death and analysed. RBC, red blood cells; WBC, white blood cells; AST, aspartate aminotransferase; ALT, alanine aminotransferase. Errors are SEM for 14–16 mice per group.

knockout experiment, which suggests that *MBD2* is not required for normal growth and viability, our data support the conclusion that the ODN antisense treatment does not result in additional unexpected toxicity to normal tissues.

In summary, *MBD2* antisense oligo knockdown has a distinct effect on tumourigenesis *in vivo* and *in vitro* but does not affect proliferation of normal and cancer cells.

## Discussion

This paper shows that pharmacological inhibition of *MBD2* blocks the growth of human cancer cells in mice *in vivo*, provides a proof of principle for *MBD2* as an anticancer target and describes the selection of a second generation *MBD2* antisense oligo as a candidate anticancer agent. It has been previously demonstrated that antisense molecules with a modified backbone can distribute widely within the body (27,42) following tail vein injection and are stable for several days. In this paper we show that tumourigenic indices both *in vitro* and *in vivo* are reduced by *MBD2* antisense oligos, which inhibit *MBD2* expression in the nanomolar range (Figure 1). Parenteral injection of small antisense molecules results in significant tumour growth retardation and even complete absence of tumours at the 8 mg/kg concentration with a minimal degree of adverse effects on blood and liver cells. One important question that should be addressed in future experiments is whether it is necessary to treat the mice three times per week with high doses of oligo or whether a short treatment will suffice and thus any possible side-effects can be avoided? It is interesting to note that a short treatment for 48 h *in vitro* was sufficient to block the ability of HCT116 cells to grow after implantation *in vivo* (Figure 3A).

The fact that pretreatment of cells with an *MBD2* antisense oligo results in inhibition of their ability to grow on soft agar (Figure 2) or implant in nude mice (Figure 3A and B), even though they were not further treated, either during their soft agar growth or their growth as xenografts *in vivo*, shows that a certain level of *MBD2* expression is required for the initial steps of tumour cells implantation *in vivo*. In addition, *MBD2* is required to enable the tumours, once implanted, to continue and survive in a non-cognate environment, as indicated by the inhibition of tumour growth by antisense treatment 3 days after implantation (Figure 3B and C) and the regression of implanted tumours following *MBD2* antisense treatment

(Figure 3E and F). It is unclear yet whether *MBD2* controls different sets of genes required for implantation and those required for continuous growth of tumours *in vivo* or whether the same proteins are involved in both processes. Genetic knockout data suggested that *MBD2* protein was required for tumourigenesis in the mouse (24) but it did not address the question of whether inhibition of *MBD2* can reverse the growth of already established tumours, which is critical if *MBD2* inhibitors are to be used as anticancer therapeutics. The data presented here support the hypothesis that *MBD2* inhibitors could serve as anticancer therapeutics. It also illustrates that *MBD2* is required for tumourigenesis of human cancer and is not an idiosyncrasy of the mouse system.

*MBD2* antisense treatment *in vitro* does not result in overt toxicity against normal proliferating cell nor does it inhibit the cell cycle of tumour cell lines, supporting the hypothesis that *MBD2* inhibition has a specific anticancer effect. This is in accordance with previously published data using either genetic knockout of the *mbd2* gene (25) or gene therapy antisense knockdown of *MBD2* (23). The lack of toxicity and normal cell growth inhibition for our agents indicates that *MBD2* antisense oligonucleotides show potential as anticancer agents and that *MBD2* is a promising and unique anticancer target, since many anticancer agents either damage cell division or target cell cycle regulatory mechanisms, inevitably resulting in toxic effects on normally cycling cells.

The unique role of *MBD2* in cancer remains to be fully defined and the genes controlled by *MBD2* that are required for tumourigenesis in either the mouse knockout model recently described (24) or in our model are as yet unknown. It is tempting to speculate that *MBD2* controls the function of genes which are specifically required to bypass normal growth inhibitory signals that are elicited when a cell is found in either anchorage-independent growth conditions or in a non-cognate environment *in vivo*. These functions might not be required for normal cell growth or normal development. The tools developed in this study will allow future experiments aimed at addressing the specific role of *MBD2* in tumourigenesis.

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**Declaration of interest:** M.Szyf is a president of Epionco Inc. in Montreal. Epionco Inc. has an option to licence MBD2 on tissues.

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